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Transforming growth factor β1 (TGF-β1) orchestrates the response of different cell types to injury via regulation of proliferation, apoptosis and ECM composition. Previously we discovered that TGF β1 is rapidly activated in mammary gland following radiation. Because TGF- β1 is implicated in regulation of proliferation and apoptosis, we investigated whether the activation of TGF-β1 contributes to the cell fate decisions in response to radiation. We found that radiation-induced apoptosis and cycle cell arrest are absent in adult mammary epithelium and embryonic liver and epidermis when TGF-β1 is compromised. Since p53 abundance and activity is thought to dictate apoptotic cellular responses to radiation, we examined the p53 response. We found that both chronic and transient depletion of TGF-β1 compromise the p53 response. In order to study the mechanism by which TGF-β1 affects the p53 response we cultured mammary epithelial cells (MECs). This in vitro model present TGF-β1 dependent radiation response similar to that seen in vivo. Treatment of MECs with TGF-β1 restored both p53 response and caspase 3 cleavage in the heterozygote cultures. We propose that TGF-β1 is a key regulator of epithelial genomic integrity since its loss impairs activation of p53 resulting in reduced apoptosis and cell cycle arrest.

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INTRODUCTION

Transforming growth factor β1 (TGF-β1) orchestrates the response of different cell types to injury via regulation of proliferation, apoptosis and ECM deposition and composition. TGF β1 is secreted as a latent complex consisting of TGF- β1 joined to the latency-associated peptide (LAP). Extracellular modifications result in the activation of TGF- β1. Previously we discovered that TGF β1 is rapidly activated in mouse mammary gland following radiation exposure. Radiation is one of the few exogenous stimuli known to cause latent TGF β1 activation. Because TGF- β1 is widely implicated in regulation of proliferation and apoptosis, we asked whether the activation of TGF-β1 contributes to the cell fate decisions in response to radiation. To do so, we used the *Tgf*β1 knockout mouse model. *Tgfb1* -/- mice die of gross inflamation at 3 weeks of age, thus precluding analysis of mammary radiation responses. *Tgf*β1 +/- mice however are viable even though there is a 70-90% reduction in TGF-β1 protein levels. These mice provide an experimental model of TGF-β1 depletion following ionizing radiation. We found that radiation-induced apoptosis in adult mammary epithelium is absent when TGF-β1 is compromised. Further *Tgfβ1* knockout embryos lack both an apoptotic and growth arrest response to radiation. Since p53 abundance and action is thought to dictate apoptotic cellular responses to radiation, we examined the p53 response as a function of chronic depletion in the *Tgfβ1* knockout mouse model and transient depletion by TGF-β1 neutralizing antibodies. Using p53 serine 18 phosphorylation (Ser-18P) as a marker of p53 stabilization in response to exogenous stress, we found that both chronic depletion and transient inhibition of TGF-β1 compromises the p53 response. Then we developed an *in vitro* model which allow us to study the mechanism by which TGF-β1 affects the p53 response. Mammary epithelial cells (MEC) derived from pregnant *Tgfβ1* null heterozygote or wildtype mice were cultured and exposed to 5 Gy on day 2 in serum free media. These cells presented TGF-β1 dependent p53 radiation response similar to that seen in mammary gland of mice. Final consequences of this TGF-β1 dependent p53 response were the ones observed *in vivo*, apoptosis and cell cycle arrest. Treatment of MECs with TGF-β1 for 4 hours before irradiation restored both p53 response and caspase 3 cleavage in the heterozygote cultures. We propose that TGF-β1 is a key regulator of epithelial genomic integrity since its loss impairs activation of p53 resulting in reduced apoptosis and cell cycle arrest.

BODY

SPECIFIC AIMS

1. To test the hypothesis that TGF-∃ is a key mediator of cellular responses to radiation

    a) To determine the potential role of TGF-∃ in modulating cellular responses to radiation we irradiated *Tgf*β1 +/+, +/- mammary epithelial cells (MECs) primary cultures and compare them respect to key cellular responses, cell cycle arrest and induction of apoptosis

    b) To confirm the role of TGF-∃ in cellular responses to radiation we added exogenous TGF-∃ to *Tgf*β1 +/- MECs in order to test if this addition would result in a response similar to that observed in the *Tgf*β1 +/+ cells.

2. To delineate individual and overlapping pathways of TGF-∃ and p53 in cellular responses to radiation

    a) To determine the potential role of TGF-∃ in modulating the p53-mediated DNA damage responses we looked at p53 phosphorylation (ser 18 and 20) and induction of downstream target genes including p21 and active caspase 3.

    b) To confirm a modulatory role of TGF-∃ in p53 activation we tested if the addition of TGF-∃ to the *Tgf*β1 +/- MECs could restore the p53 response.

3. To dissect out overlapping pathways of TGF-∃ and p53 in radiation responses of wholebody-irradiated mice

    a) To determine the role of TGF-∃ in the modulation of radiation responses. We irradiated *Tgf*β1 +/+, +/- mice and compare them respect to cellular responses, cell cycle arrest and induction of apoptosis.

    b) To delineate the role of TGF-∃ in modulating the p53-mediated DNA damage response we compared the levels of p53 Ser 18 and p53 Ser 20

c) To confirm the modulatory role of TGF-∃ in p53 activation both chronic and transient TGF∃1 depletion was used to test if p53 phosphorylation is inhibited.

PROGRESS

Dr. Pajares returned to her home in Spain in December, 2003 for personal reasons. She has since assumed a position in the Department of Histology and Pathology at the School of Medicine in the University of Navarra (Pamplona, Spain). Since that time, the fellowship has been dormant. Although we have made progress on this project, we have not used the funding provided by this fellowship since an appropriate candidate was not available. In light of this history, we wish to relinquish this funding. *The summary below is from last year's report.*

This fellowship was transferred to Dr. Pajares in January 2002 because Dr. Mukherjee, the fellow who had been awarded the fellowship, left the laboratory for another position in 2001. In the meantime, Dr. Barcellos-Hoff had already begun the project under the auspices of a NASA funded proposal entitled "Interactions between tissue and cellular stress responses following charged particle exposure", which was the source of the preliminary data in the original proposal. Dr. Pajares began as the laboratory was finishing what essentially amounts to the Specific Aim 3 in this proposal. She contributed to this by making mammary gland tissue extracts and analyzing p53 by immunoblotting, which earned her a co-authorship on the publication of these data in Cancer Research in October, 2002 [Ewan, 2002]. These data are summarized below, under Aim 3. She then focused on developing cell culture models and conducting experiments to test the hypotheses in Aim 1 and Aim 2. The abstract of these studies for the ERA of HOPE meeting in Orlando was selected for a platform presentation by Dr. Pajares. A second publication is under preparation, for which Dr. Pajares will be first author.

AIM 1

Mammary epithelial from *Tgf*β heterozygote mice fail to undergo cell cycle arrest or apoptosis in response to irradiation.

In order to test the potential role of TGF-∃ in modulating cellular responses to radiation we developed primary mammary epithelial cells (MECs) cultures model. We generated MECs primary cultures from mammary glands of adult *Tgf*β1 wildtype and heterozygote mice and irradiated them. Because the number of cells was limited, we used protein extraction and immunoblotting instead of cellular characterization assays (i.e. TUNEL and flow cytometry) as proposed in the first statement of work. The role of TGF-∃ in cell cycle regulation following irradiation was studied by determining the levels of p21 by western blot instead of flow cytometric analyses. Levels of p21, a protein related to cell cycle arrest, were increased in TGF-∃ wildtype MECs 4 hours after irradiation, TGF-∃ heterozygote cells, however, did not present as high increase as the one seen in the wildtype

ones (Fig 1). These results showed that TGF-∃ is regulating cell cycle arrest in response to irradiation. The apoptosis response was assayed by studying of the levels of active caspase 3 by western blot instead of TUNEL staining. Activation of caspase 3 is an early event in most of the apoptotic pathways. Active caspase 3 levels peaked by 4 hours postirradiation in TGF-∃ wildtype MEC, but were reduced in the heterozygote MEC (Fig 2). These findings mean that TGF-∃ is regulating induction of apoptosis as well as cell cycle arrest in irradiated primary mammary epithelial cells.

AIM 2

Rapid modifications of p53 that lead to its stability and activity are decreased in *Tgf*β1 heterozygote MEC.

We observed that TGF∃1 mediated key cellular responses to irradiation in the MECs, so we tested our hypothesis that p53 and TGF∃1 mediated signaling pathways might intersect. In order to do that we studied the p53 activation status determined by protein modification, we used two different phosphorylation state-specific antibodies. Phosphorylation of both Ser-18 (Ser-15 in human) is strongly associated with the cellular response to radiation damage (e.g. apoptosis, cell cycle block) and contribute to stabilization and activation of p53 protein. Immunoblotting of cellular protein extracts showed that previous to irradiation, levels of p53 Ser 18-P (Fig 3) were low in extracts from both TGF∃1 wildtype and heterozygote cells but were significantly elevated within 1 hour of irradiation exposure. At 2 and 4 hours post irradiation, the levels of p53 Ser 18-P still remain detectable in both genotypes. Thus, TGF∃1 modulates the activation of p53, which in turn is known to mediate DNA damage responses in mammary epithelial cells.

In order to test whether in other types of cells the p53 radiation response is TGF∃1 dependent, we developed a mouse embryonic fibroblast (MEF) model. We compared the p53 response postirradiation of +/+, +/-, -/- MEFs cultures from embryos coming from *Tgf*β1+/- mice. Neither *Tgf*β1 null nor heterozygote irradiated MEF exhibit reduced Ser-18P compared to wildtype (Fig 4). Thus, TGF∃1 dependent p53 phosphorylation appears to be epithelial specific, which is further supported by our in vivo data reported below.

TGF-β treatment restores p53 phosphorylation and apoptotic response in *Tgfβ1* heterozygote MEC.

We propose that TGF-β is a key regulator of epithelial genomic integrity since its loss impairs activation of p53, resulting in reduced apoptosis and cell cycle arrest. In order to confirm the modulatory role of TGF-∃ in p53 activation, we treated *Tgf*β1 +/- MECs cultures with TGF∃1 for 4 hours before IR restored both the p53 response and the caspase-3 cleavage in the heterozygote MEC (Fig 5) .

AIM 3

We tested the hypothesis that TGF∃1 modulates the type and degree of cellular damage responses *in situ*. The decision of a cell to undergo apoptosis in response to DNA damage is commonly attributed to the level of DNA damage and certain cellular competencies that are poorly understood *in vivo*. The data reported here reveal a surprising TGF-∃ dependence for cellular response to DNA damage. Upon finding that radiation-induced apoptosis was undetectable in *Tgf∃1* +/- mammary gland, we examined the apoptotic response in embryonic tissues as a function of *Tgf∃1* gene dosage. Radiation-induced apoptosis correlated with TGF-∃ abundance in both liver and epidermis. In addition, the IR-induced proliferative block was completely absent in irradiated *Tgf∃1* null embryo tissues. Since both responses have been shown to be p53-dependent (1-3), we then examined the phosphorylation of Ser-18 associated with rapid p53 activation (4). Depletion of TGF∃1 abrogated p53 phosphorylation in mammary glands in both the chronically depleted knockout mice or following transient inhibition using TGF∃1 neutralizing antibodies

TGF∃1 activation and activity are reduced in mammary glands of irradiated *Tgf∃1* +/- mice.

We have confirmed that TGF∃1 protein levels of *Tgf∃1* +/- adult mammary gland are reduced by more than 90% compared to wildtype (5). To determine whether IR-induced TGF∃1 activation was also compromised, we localized active TGF∃1 by immunostaining. Active TGF∃1 was greatly reduced in irradiated *Tgf∃1* +/- mammary gland compared to wildtype tissue (Figure 6A). To confirm that depletion of TGF∃1 resulted in decreased TGF∃1 signaling, we examined the induction of Smad 2/3 nuclear translocation. A marked induction of nuclear Smad 2/3 immunostaining was observed 1 h in irradiated versus sham-irradiated wildtype mice (Figure 6B). The frequency of positively stained cells and the intensity of staining were reduced in irradiated *Tgf∃1* +/- mammary epithelium, indicating that *Tgf∃1* +/- mice are an appropriate model to study whether TGF∃1 depletion affects cell fate decisions.

Radiation-induced apoptosis is absent in *Tgf∃1* +/- mammary epithelium.

Previous studies demonstrated that a dose of 5 Gy IR induces a 2-3 fold increase in apoptosis that peaks at 6 h in mammary gland of nulliparous animals (1, 2). During studies of mammary development, we observed that the background frequency of apoptosis is related to the stage of estrus cycle and that both proliferation and apoptosis peaks at estrus (5). To ensure comparable background frequency, the animals were irradiated in estrus. The apoptotic index increased 3-fold in mammary glands of C57BL/6/129Sv *Tgf∃1* +/+ mice 6 h following whole body exposure to a dose of 5 Gy (-radiation (Figure 7A). In contrast, mammary epithelial apoptosis was not significantly increased following irradiation of *Tgf∃1*+/- mice and was in fact 1/8th the level of irradiated wildtype mice. Although physiological apoptosis in *Tgf∃1* +/- mammary epithelium at estrus is half that of

wildtype mice, apoptosis is not generally depressed in *Tgf∃1* +/- mammary epithelium since levels are similar to wildtype at puberty and is increased during pregnancy (5). In addition, radiation-induced apoptosis in lymph node and spleen was similar in *Tgf∃1* +/- mice and wildtype mice (not shown). These data suggest that TGF∃1 affects cell fate decisions in response to DNA damage in a cell type-dependent manner.

Absence of TGF∃1 in embryonic tissues abrogates apoptotic and cell cycle inhibition in response to IR.

The radiation response of adult TGF∃1 null mice cannot be determined because *Tgf∃1* -/- genotype mice commonly die *in utero* (6). However, several embryonic tissues exhibit both a robust apoptotic response and cell cycle inhibition shortly after irradiation in utero (3). Therefore 12.5 d pregnant *Tgf∃1* +/- dams were irradiated whole body with a dose of 5 Gy and the embryos collected 6 h later. Apotag- positive cells were counted in epidermis and liver (Figure 7 B,C). Apoptosis increased 2-3 fold in epidermis and liver in irradiated wildtype embryos. Radiation-induced apoptosis was significantly decreased in *Tgf∃1* +/- embryos. *Tgf∃1* -/- embryos lacked an apoptotic response.

In rapidly proliferating tissues, IR can also induce a transient cell cycle block. Antibodies to PCNA were used to define the frequency of cells in cycle in embryonic tissues following IR (Figure 7 D,E). Proliferation was reduced 2-3 fold following irradiation in liver and epidermis of both +/+ and +/- embryos. The frequency of proliferating cells was unaffected in irradiated -/- embryos. Together, these data demonstrate that TGF∃1 abundance dictates cell fate decision in irradiated embryonic as well as adult epithelial tissues.

p53 stress response is activated in irradiated mammary gland.

Apoptosis is p53 dependent in irradiated mammary gland and embryos (1-3). However, a recent report suggested that mammary gland lacks a classic p53 IR induction as measured by nuclear immunoreactivity using the CM5 antibody (2). Since this antibody many be insensitive to p53 activation status determined by protein modification, in the current study we used a phosphorylation state-specific antibody. (((Phosphorylation of Ser-18 (Ser-15 in human) is strongly associated with the cellular response to radiation damage (e.g. apoptosis, cell cycle block) and contributes to p53 protein stability (4). The phosphorylation of Ser-18 promotes dissociation of p53 from the MDM2 protein, which otherwise directs p53 proteolysis)))) Immunoblotting of total mammary gland protein extracts showed that Ser-18P was undetectable in extracts from sham-irradiated tissue. Within 1 h of IR exposure Ser-18P was significantly elevated and remained detectable up to 24 h following IR (Figure 8A). Total p53 levels, detected using antibodies PAb122 or CM1, which are insensitive to phosphorylation status, were increased at 24 h post-IR, but unchanged during the period from 1-15 h (data not shown).

Since the mammary gland is comprised of many cell types, we used immunofluorescence to determine the cellular localization of p53 bearing Ser-18P (Figure 8B). Mammary epithelium from sham-irradiated mice showed minimal nuclear signal. The immunoreactivity of phospho-specific p53 Ser-18P antibodies was restricted to the nucleus and was punctate in irradiated mammary epithelial nuclei. Epithelial nuclear p53

Ser-18P immunostaining was significantly increased within an hour of radiation exposure and remained prominent up to 24 h after irradiation.

Chronic or transient TGF∃1 depletion inhibits p53 Ser-18 phosphorylation.

Immunoblots of p53 Ser-18P using total protein extracts from wildtype mice showed a massive induction of p53 phosphorylation 1 h post-IR (Figure 9A). Between 1 and 6 h, Ser-18P p53 levels decreased approximately 10-fold in wildtype mice, but were still elevated compared to sham. In contrast, Ser-18P detection was decreased at least 4-fold at both 1 h and 6 h compared to wildtype mice. Total p53 in wildtype and heterozygote mammary extracts measured by CM1 or CM5 were similar (data not shown), suggesting that phosphorylation, rather than abundance, was severely and persistently compromised. The immunolocalization of p53 Ser-18P in irradiated *Tgf∃1* +/- mammary epithelium was also compared to that of wildtype mice (Figure 9B). Nuclear p53 Ser-18P immunofluorescence was significantly reduced at 1 h post-irradiation in the *Tgf∃1* heterozygote compared to wild type mammary epithelium. The difference between irradiated wildtype and *Tgf∃1* +/- mice was less pronounced at 6 h post-irradiation but was still attenuated in *Tgf∃1* +/- mammary epithelial cells.

Chronic depletion in *Tgf∃1* +/- mice could perturb aspects of cell physiology that modify the p53 radiation response. To test whether TGF∃1 directly affected the radiation response, pan-specific TGF∃1 neutralizing antibodies were administered i.p. 3 h before irradiation. Our previous studies had demonstrated the efficacy of this timing, route and antibody dose in blocking TGF∃1 mediated extracellular matrix remodeling (7). Immunoblotting showed that p53 Ser-18P after radiation exposure was reduced from neutralizing antibody treated mice compared to animals treated with control antibody (Figure 9C). p53 Ser-18P was reduced 5-fold at 1 h post-IR in animals receiving TGF∃ neutralizing antibody compared to those receiving control antibody. This difference was less evident at 4 h post-irradiation. Likewise, nuclear localization of p53 Ser-18P determined by immunofluorescence staining was significantly reduced 1hr after IR when TGF∃1 was transiently depleted prior to irradiation (Figure 9D). As seen in the gene knockouts, TGF∃1 neutralizing antibody treatment did not alter levels of total p53, as detected using either CM1 or CM5 antibody (data not shown), indicating that TGF∃ availability affected p53 post-translational modification rather than total protein abundance.

In light of the very promising results showing that inhibition of TGF∃1 prevents p53 radiation responses, we have decided to move forward with experiments to examine the mechanism by which TGF∃ is affecting the p53 response. We have done previos experiments that show that TGF beta could be affecting ATM kinase, protein that phosphorilate p53 directly at Ser 18 and indirectly, through chk2, at Ser 20. We will determine if TGF∃ is affecting the levels of ATM kinase, its activity or both.

KEY RESEARCH ACCOMPLISHMENTS

- TGF∃1 activation and activity are reduced in mammary glands of irradiated *Tgf∃1* +/- mice respect the wildtype ones.

- Radiation-induced apoptosis is absent in *Tgf∃1* +/- mammary epithelium.

- Absence of TGF∃1 in embryonic tissues abrogates apoptotic and cell cycle inhibition in response to IR.

- Radiation induces p53 Ser 18 phosphorylation mammary gland.

- Radiation-induced p53 Ser-18 phosphorylation is decreased in the mammary gland of chronically or transiently TGF∃1 depleted mice.

- Primary mammary epithelial cells represent good in vitro model to study the mechanism by which TGF∃1 affects the p53 response. p53 and apoptotic responses are reduced in *Tgf∃1* +/- MECs.

- Levels of p21 are reduced in the *Tgf∃1* heterozygote cells.

- Addition of exogenous TGF∃1 restores p53 and apoptotic response.

- Neither *Tgf∃1* +/+ nor +/- mouse embryonic fibroblast (MEFs) exhibit differences in the levels of p53 Ser 18P compared to wildtype ones.

REPORTABLE OUTCOMES

1) Paper titled "Transforming growth factor-∃1 mediates cellular response to DNA damage *in situ*" was published in Cancer Research 62, 5627-31 in October 15, 2002.
2) Abstract titled "Transforming growth factor-∃1 regulates radiation induced apoptosis and p53 response" for a poster presented at the Mammary gland Gordons conference that took place in Lucca (Italy) in April 28-May 3, 2002.
3) Abstract titled "Transforming growth factor-∃1 regulates radiation induced apoptosis and p53 response" for a poster presented at the Era of Hope meeting that took place in Orlando (Florida) in September 25-30, 2002.

CONCLUSIONS

Using the *Tgf β1* null heterozygote model we have determined that TGF-β1 dictates epithelial cell fate in response to DNA damage. After IR, there is a rapid and persistent increase in p53 Ser-18P in the mammary gland epithelium. The phosphorylation of p53 at Ser-18 is reduced when TGF-β1 levels are compromised, either chronically or transiently. Primary mammary epithelial cells present a TGF-β1 dependent p53 response post-irradiation. Levels of p53 Ser 18-P were decreased in the TGF- β1 heterozygote MECs respect to the wild type MEC. Apoptosis and cell cycle arrest were also compromised. In contrast, mouse embryonic fibroblast TGF- β1 null and heterozygote cells do not show reduced p53 Ser 18 compared to wild type. Addition of TGF- β1 to heterozygote mammary epithelial cells restores p53 and apoptotic responses.

REFERENCES

1. Meyn, R. E., Stephens, L. C., Mason, K. A., and Medina, D. Radiation-induced apoptosis in normal and pre-neoplastic mammary glands in vivo: significance of gland differentiation and p53 status. Int J Cancer, *65:* 466-472, 1996.
2. Kuperwasser, C., Pinkas, J., Hurlbut, G. D., Naber, S. P., and Jerry, D. J. Cytoplasmic sequestration and functional repression of p53 in the mammary epithelium is reversed by hormonal treatment. Cancer Research, *60:* 2723-2729, 2000.
3. Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy [see comments]. Science, *285:* 1733-1737, 1999.
4. Chao, C., Saito, S., Anderson, C. W., Appella, E., and Xu, Y. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. Proc Natl Acad Sci U S A, *97:* 11936-11941, 2000.

5. Ewan, K. B., Shyamala, G., Ravani, S. A., Tang, Y., Akhurst, R. J., Wakefield, L., and Barcellos-Hoff, M. H. Latent TGF-∃ activation in mammary gland: Regulation by ovarian hormones affects ductal and alveolar proliferation. Am J Path, *160:* 2081-2093, 2002.

6. Letterio, J. J., Geiser, A. G., Kulkarni, A. B., Roche, N. S., Sporn, M. B., and Roberts, A. B.
Maternal rescue of transforming growth factor-∃1 null mice. Science, *264:* 1936-1938, 1994.

7. Ehrhart, E. J., Carroll, A., Segarini, P., Tsang, M. L.-S., and Barcellos-Hoff, M. H. Latent transforming growth factor-∃ activation in situ: Quantitative and functional evidence following low dose irradiation. FASEB J, *11:* 991-1002, 1997.

APPENDICES

Appendix I: Figure legends and figures

Appendix II: Paper published in Cancer Research.

Ewan KB, Henshall-Powell RL, Ravani S, Pajares MJ, Arteaga C, Warters R, Akhurst, RJ and Barcellos Hoff MH "Transforming growth factor ∃1 mediates cellular response to DNA damage *in situ*" in cancer research 62, 5627-31, 2002.

APPENDIX I

FIGURE LEGENDS

Figure 1: TGF-∃1 blocks cell cycle in response to radiation. Primary mammary epithelial cells derived from pregnant Tgf-∃ null heterozygote mice (HT) or wildtype mice (WT) were cultured and exposed to irradiation (IR) (5 Gy) on day 2 in serum free media. Immunoblots show that p21 protein increased in the wildtype mammary epithelial cells 4 hours after IR. p21 was significantly reduced 4h after IR in ***TGF-∃1*** *+/-* cells compared to the wildtype ones.

Figure 2: Irradiated *TGF-∃1* +/- primary mammary epithelial cells show reduced levels of caspase 3 cleavage. Western blot of cell extract from wild type or ***TGF-∃1*** heterozygote primary mammary epithelial cells, sham and 4 h after IR (5 Gy). Antibodies to 17 KD fragment of caspase 3 were used in Western blotting of extract of ***TGF-∃1*** +/- and +/+ primary mammary epithelial cells as an index of apoptosis. Activation of caspase 3 through cleavage occurs in most of the apoptotic pathways. 17 KD Caspase 3 protein was highly increased in wildtype cells 4 hours following irradiation, however the increase is not so pronounced in the heterozygote cultures.

Figure 3: The p53 response is reduced in *TGF-∃1* +/- primary mammary epithelial cells. Antibodies to p53 Ser-18P were used in Western blotting of protein extract of ***TGF-∃1*** +/- and +/+ primary mammary epithelial cells. Levels of p53 Ser 18P were really low in the sham-irradiated cells from both wildtype and heterozygote genotype. Within 1 hour following irradiation, p53 Ser 18P was significantly increased, decreasing after the first hour posIR. 2 and 4 hours after IR we still observed higher levels of p53 Ser 18 in the wild type cells than in the heterozygote ones.

Figure 4: *TGF-∃1* -/- and +/- mouse embryonic fibroblast do not show a reduced p53 response following irradiation. *TGF-∃1* +/+, +/-, and -/- mouse embryonic fibroblast (MEF) were cultured in a sera free media and irradiated (5 Gy). Western blot of protein extract 1 hour posIR showed that p53 Ser-18P increase within one hour of irradiation but the levels of this protein were not reduced in the irradiated *TGF-∃1* +/- and -/- MEFs as in mammary epithelial cells.

Figure 5: Addition of exogenous TGF-∃1 restores p53 and apoptotic response. TGF-∃1 at a dose of 500 pg/ml was added to the *TGF-∃1* +/- 4 hours before IR. **(A)** The levels of p53 Ser-18P were measured by inmunoblot in the *TGF-∃1* +/- cells 1 hour after IR and compared with the non-treated *TGF-∃1* +/+. TGF-∃1 treated cells presented similar levels of p53 Ser 18, 1hour, 2 and 4h postIR than the wild type ones. **(B)** TGF ∃1 treated *TGF-∃1* heterozygotes cells presented higher levels of 17 KD fragment of caspase 3 than the non treated ones, these levels were similar to the *TGF-∃1*wild type cells.

Figure 6: Irradiated *TGF-∃1* +/- mammary gland shows reduced levels of active TGF-∃1 and Smad 2/3. *TGF-∃1* +/- and +/+ mice were irradiated whole body to a dose of 5 Gy and sacrificed 1 h later. **(A)** False color digital micrographs of dual immunofluorescence of antigen-purified TGFβ1 antibody (red) and LAP antibody (green) visualized simultaneously with DAPI stained nuclei (blue). Comparison of mammary gland tissue from irradiated *TGF-∃1* +/+ and +/- mice indicates that TGF-∃1 immunoreactivity (yellow-orange) is greater in *TGF-∃1* +/+ mice, while *TGF-∃1*+/- mice show predominant LAP immunoreactivity (green). The prominent localization of TGFβ1 in the irradiated wildtype mice reflects radiation-induced activation (5). Note that all cells stain with antibodies to LAP prior to radiation exposure (7). **(B)** False color digital micrographs of Smad 2/3 antibody (green) localized simultaneously with DAPI stained nuclei (blue). Comparison of mammary gland cryosections from sham (a, c) or irradiated (b, d) *TGF-∃1*+/+ (a, b) and +/- (c, d) mice indicates that IR induced significant Smad2/3 immunoreactivity. Immunofluorescence intensity was markedly reduced in irradiated *TGF-∃1* +/- mice.

Figure 7: *TGF-∃1* gene dosage correlates with reduced apoptosis and cell cycle block in response to radiation. (A) The frequency of apoptotic nuclei detected using TUNEL reaction was determined in the mammary epithelium of *TGF-∃1* +/- and +/+ mice (mean ± SEM; n= 3 animals). Sham-irradiated (black) and whole-body irradiated (gray) wildtype were significantly different (t-test; P=0.02). The irradiated *TGF-∃1* heterozygote mice were not significantly different from sham-irradiated heterozygote mice, but were significantly different from irradiated wild type (t-test; P=0.006). Pregnant NIH/OlaHsd *TGF-∃1* +/- dams were irradiated whole body (5 Gy) on day 12.5 of gestation. Embryos irradiated in utero were collected 6 h after irradiation. Apoptotic nuclei were detected using the TUNEL reaction in liver **(B)** and epidermis **(C)** from *TGF-∃1* +/+, +/- and -/- embryos. Apoptosis was decreased in control *TGF-∃1* +/- and -/- embryo tissues.

Significantly increased apoptosis was absent from both liver and epidermis of irradiated *TGF-∃1* +/- and -/- embryos. The frequency of cycling cells was detected using PCNA antibodies in sham-irradiated (black) and irradiated (gray) embryos. Radiation induced cell cycle block was evidenced by a 2-3 fold reduction of PCNA positive cells following irradiation in utero in the liver **(D)** and epidermis **(E)** from *TGF-∃* +/+ and +/- embryos. The frequency of PCNA positive cells was not significantly different between sham and IR embryos of -/- genotype, indicating abrogation of radiation-induced cell cycle block.

Figure 8: p53 Ser-18P is induced in irradiated mammary epithelial cells. (A) Antibodies to Ser-18P p53 were used in Western blotting of total tissue protein extracts of irradiated Balb/c mammary tissue. No signal was evident in sham-irradiated tissue. A single band was detected at 1 h and was present up to 24 h following radiation exposure by Western. **(B)** False color images of immunofluorescence localization of p53 Ser-18 phosphorylation detected using secondary antibodies labeled with Alexa 488 (appears green/turquoise). Nuclei were counterstained with DAPI (blue). Immunofluorescence was absent from controls in which the primary antibody was deleted (a) and discernable in only a few epithelial cells in sham irradiated tissue (b). Prominent nuclear immunoreactivity was evident throughout the epithelium from 1 h (c), 4 h (d), 15 h (e) and 24 h(g) after radiation exposure .

Figure 9: Decreased radiation-induced p53 Ser-18 P following irradiation and chronic or transient TGF-∃1 depletion. (A) Western blot of tissue extracts from wildtype or *Tgf∃1* heterozygote mice sham and 1-6 h post-IR. p53 Ser-18 phosphorylation was significantly reduced in *Tgf∃1* +/- mice 1-6 h after IR. **(B)** p53 Ser-18P was localized as indicated in Figure 3 using cryosections of C57BL/6/129Sv *TGF-∃1* +/+ mice (a, b, c) or *TGF-∃1* +/- mice (d, e, f) subjected to sham exposure (a, d) or irradiated with 5 Gy, 1 h (b, e) or 6 h (c, f) before sacrifice. Nuclear localization of p53 Ser-18P 1 h post-IR was significantly reduced in *TGF-∃1* +/- animals compared to wildtype animals. By 6 hr, p53 Ser-18P was decreased in both genotypes. **(C)** Western blot of tissue extracts from Balb/c adult female mice injected i.p. with an irrelevant IgG antibody as a control (C) or TGF-∃1 neutralizing (N) antibody prior to irradiation. p53 Ser-18P was significantly reduced 1 h after IR when TGF-∃1 neutralizing antibodies were administered before irradiation. **(D)** Nuclear immunolocalization of p53 Ser-18P was significantly reduced in animals treated with TGF-∃ neutralizing antibody. Mice received control (a, b) or TGF-∃ pan-isoform neutralizing monoclonal antibody (c, d) 3 h before sham exposure (a, c) or whole body irradiation with 5 Gy (b, d). p53 Ser-18P was localized in cryosections as indicated in Figure 8.

FIGURES

Figure 1

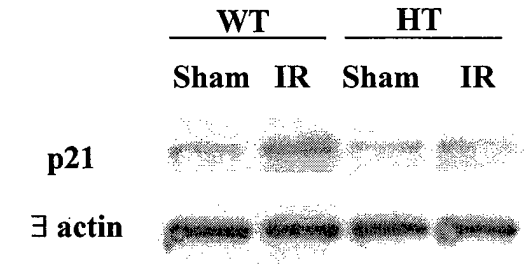


Figure 2

Figure 3

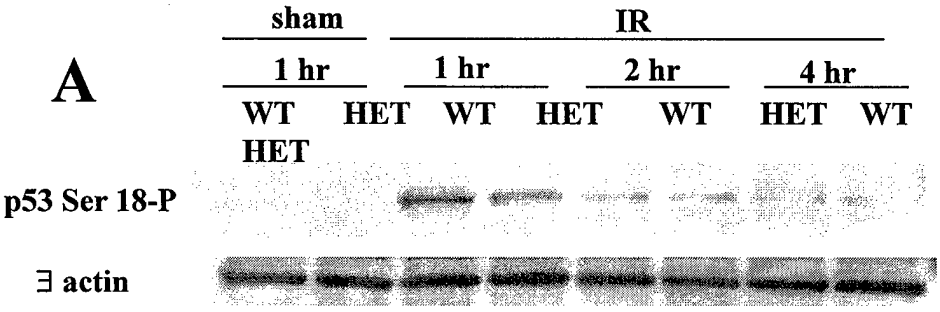


Figure 4

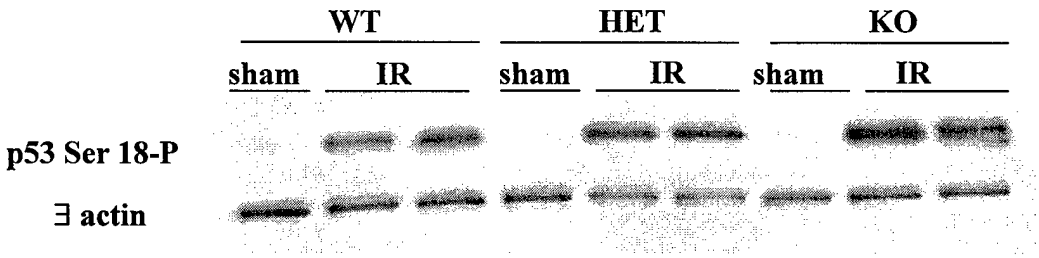
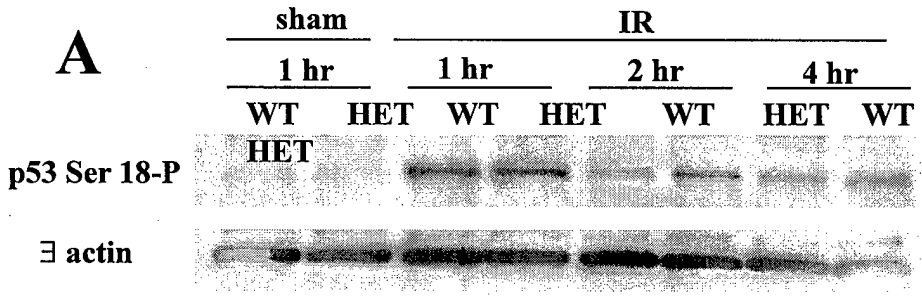


Figure 5

A



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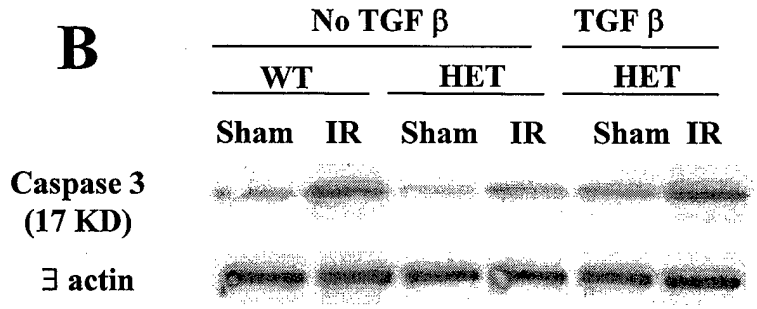
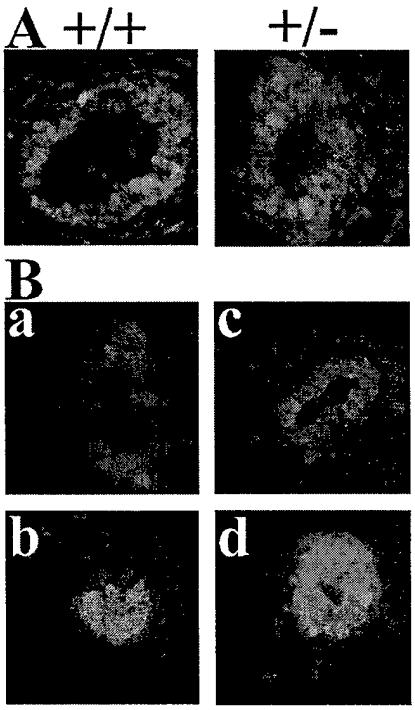


Figure 6



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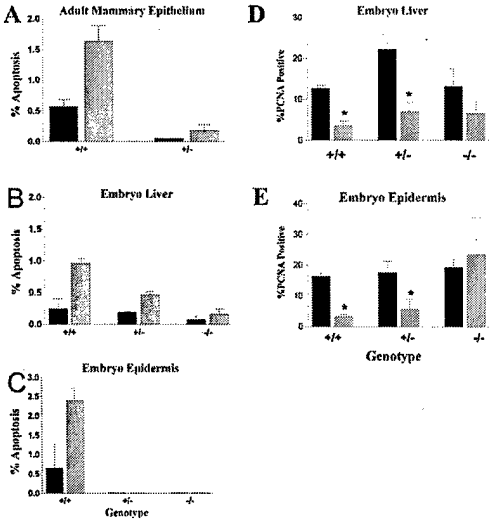


Figure 8

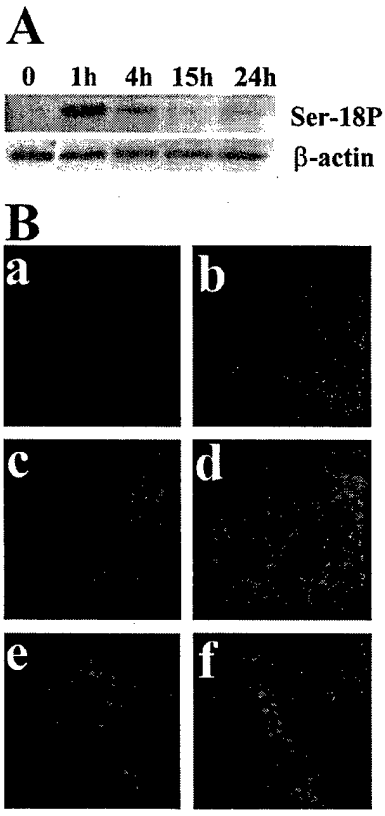
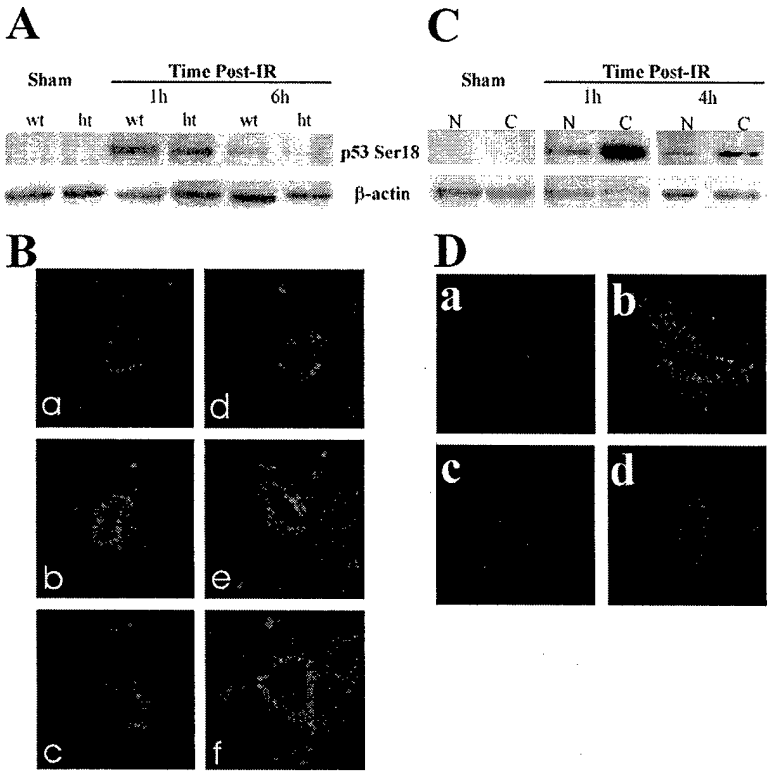


Figure 9



APPENDIX II

Ewan KB, Henshall-Powell RL, Ravani S, Pajares MJ, Arteaga C, Warters R, Akhurst, RJ and Barcellos Hoff MH "Transforming growth factor ∃1 mediates cellular response to DNA damage *in situ*" in cancer research 62, 5627-31, 2002.

Radiation-Induced Apoptosis and p53 Response Depend on Transforming Growth Factor-∃1 Levels

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FIGURES: 5

PAGES:

KEYWORDS: mouse models, mammary gland, apoptosis, cancer, radiation, breast, cytokines

RUNNING TITLE: TGF∃1 mediates radiation responses

ABBREVIATIONS: TGF∃1, transforming growth factor ∃1; IR, ionizing radiation; LAP, latency associated peptide; Ser-18P, p53 serine 18 phosphorylation; PCNA, proliferating cell nuclear antigen; DAPI, 4=,6-diamidino-2-phenylindole

ABSTRACT

Transforming growth factor-∃1 (TGF∃1) orchestrates cell fate decisions in development, differentiation and disease. We previously showed that TGF∃1 is rapidly activated in response to ionizing radiation. To test whether TGF∃1 mediates cell fate decisions following radiation exposure, we irradiated *Tgf∃1* null heterozygote mice in which levels of TGF∃1 are reduced by 90%. In wildtype mice, mammary epithelial apoptosis was increased 3-fold 6 h following whole body radiation (5 Gy) exposure. This increase was absent from irradiated *Tgf∃1* +/- mammary gland. To further examine the effect of gene dosage on cellular radiation responses, *Tgf∃1* +/+, +/- and -/- embryos were irradiated *in utero*. Relative to wildtype embryos, apoptosis was significantly reduced from irradiated *Tgf∃1* +/- embryos and was absent from *Tgf∃1* -/- embryos, which also lacked a radiation-induced cell cycle block. Since apoptosis in these tissues is p53 dependent, we next examined the mammary p53 response. In wildtype mice, phosphorylation of Ser-18 p53, an early event stabilizing p53, was induced from 1-24 h following radiation and was localized to mammary epithelial nuclei. The level of phosphorylated Ser-18 p53 was markedly reduced in irradiated *Tgf∃1* +/- mammary gland at 1 h post-irradiation compared to wildtype mice. To test whether transient, in contrast to chronic, depletion of TGF∃1 was sufficient to compromise p53 response, neutralizing antibodies were administered shortly before irradiation, which also reduced p53 Ser-18 phosphorylation at 1 h post-irradiation. Thus, TGF∃1 dictates cell fate decisions following DNA damage and these decisions correlate with changes in p53 phosphorylation.

Introduction

Transforming growth factor ∃1 (TGF∃1) orchestrates the response of multiple cell types to injury via its broad regulation of proliferation, apoptosis and ECM deposition and composition [Roberts, 1988 #428]. Resistance to TGF∃ growth inhibitory effects is a common feature of human breast, ovarian, and gastrointestinal cancer cells and genetic mutations leading to loss of TGF∃ signaling predispose certain tissues to develop cancer [Massague, 2000 #2847].

The biological activity of secreted TGF∃ is constrained by its production as a latent complex consisting of TGF∃1 non-covalently associated with its processed N-terminal pro-segment, called the latency-associated peptide (LAP). Post-translational modifications are critical regulatory events for TGF∃1 function *in vivo*: release from LAP is a prerequisite for TGF∃1 to bind to its cell surface receptors [Miyazono, 1991 #533]. This activation event acts as the switch to initiate tissue response to damage in several physiological processes including inflammation, wounding and angiogenesis [Kehrl, 1991 #2050; Wahl, 1994 #1696]. We discovered that TGF∃1 is rapidly activated in mouse mammary gland following radiation exposure [Barcellos-Hoff, 1994, #577]. Radiation is one of the few exogenous stimuli known to cause latent TGF∃1 activation *in situ* [Barcellos-Hoff, 1994, #577; Ehrhart, 1997 #1879]. Consistent with increased active TGF∃1, treatment with TGF∃ neutralizing antibodies inhibit mammary gland extracellular matrix remodeling in irradiated mice [Ehrhart, 1997 #1879]. We have shown that latent

TGF∃1 activation can occur via free radical generation by radiation and other sources, thus endowing TGF∃1 with the ability to act as an extracellular sensor of oxidative stress [Barcellos-Hoff, 1996 #1871].

TGF∃'s pleiotropic actions are well-suited to orchestrate cellular responses to radiation damage and to facilitate reestablishment of homeostasis by eliminating damaged cells or promoting repair. Because TGF∃ is widely implicated in regulation of proliferation and apoptosis, we asked whether the activation of TGF∃ contributes to the cell fate decisions in response to radiation. To do so, we used the *Tgf∃1* knockout mouse model. *Tgf∃1* -/- mice die of gross inflammation at 3 weeks of age, thus precluding analysis of mammary radiation responses [Letterio, 1994 #1867]. However, *Tgf∃1* +/- mice are viable despite a 70-90% reduction in TGF∃ protein levels [Tang, 1998 #2316]. These mice provide an experimental model of TGF∃ depletion.

We found that radiation-induced apoptosis in adult mammary epithelium is absent when TGF∃ is depleted. Further *Tgf∃1* knockout embryos lack both an apoptotic and growth arrest response to radiation. Since p53 abundance and action is thought to dictate apoptotic cellular responses to radiation [Agarwal, 1998 #3415], we next examined the p53 response as a function of chronic depletion in the TGF∃1 knockout mouse model and transient depletion by TGF∃ neutralizing antibodies. Using p53 serine 18 phosphorylation(Ser-18P) as a marker of p53 stabilization in response to exogenous stress, we found that both chronic depletion and transient inhibition of TGF∃1 compromises the p53 response. These data, together with our previous observations [Barcellos-Hoff, 1994 #577; Ehrhart,1997 #1879], suggest that TGF∃1 signaling, controlled via the extracellular activation of latent TGF∃1, is a critical mediator of cellular responses to radiation.

Materials and Methods

Adult 129Sv/C57BL/6 *Tgf∃1* +/+ or +/- mice were generously provided by Dr. Anita Roberts (National Cancer Institute, NIH). Genotyping was performed by PCR as described [Tang, 1998 #2316]. DNA was extracted using the GenomicPrep cell and tissue DNA isolation kit (Amersham Pharmacia, Piscataway NJ), according to the manufacturer's instructions. HotStarTaq (Qiagen, Valencia CA) Taq DNA polymerase was used and oligonucleotides were custom made by Operon (Alameda, CA). Pregnant NIH/OlaHsd *Tgf∃1* +/- dams were irradiated on day 12.5 of gestation, timed from observation of the vaginal plug. Embryos were dissected from the uterus 6 h after irradiation. Placental and tail tissue was digested for genotyping with Proteinase K. In some experiments, adult BALB/c mice (B&K, Fremont, CA) were injected i.p. 3 h before irradiation with 0.5 :g of pan-specific TGF∃1 neutralizing antibody 2G7 purified IgG2b [Koli, 1997 #2223] or irrelevant immunoglobulin-matched antibody (Sigma Pharmaceuticals, St. Louis, MO). Unanesthetized adult mice were irradiated whole body with ^{60}Co (-irradiation using a dose rate of 24 cGy/min to total dose of 5 Gy. Dosimetry was determined using a Victoreen ionization chamber prior to each experiment. Estrus was staged using cytological characteristics of vaginal smears at the time of irradiation and confirmed postmortem by uterine wet weight. Animals from each group were euthanized by CO_2 inhalation and cervical dislocation at the indicated times in accordance with AAALAC guidelines and with institutional review and approval. The inguinal mammary glands were embedded for

histology immediately after dissection. Protein extracts were prepared from the 3rd and 5th glands that were flash-frozen in liquid nitrogen.

Immunofluorescence: Tissues or embryos were embedded in Tissue-Tek^R compound (Sakura Finetek U.S.A., Inc., Torrance, CA), immediately frozen in a dry ice/ethanol bath, and stored at -80EC. 5:m cryosections were cut at -30EC onto gelatin-coated coverslips. Immunostaining to differentiate between active and latent TGFβ1 was conducted as previously described [Ehrhart, 1997 #1879]. Sections were fixed using 2% buffered paraformaldehyde followed by a 0.1 M glycine/PBS wash for the following antibodies: goat anti-latency associated peptide (LAP, R&D Systems, Minneapolis, MN), chicken anti-TGFβ1 antibodies (AF-101-NA, Lots # FS03 and # FS08, R&D Systems), Smad 2/3 (FL-425, Santa Cruz Biotechnology Inc), and proliferating cell nuclear antigen (PCNA) fluorescein-conjugated monoclonal antibodies (DAKO, Carpinteria, CA). For PCNA, paraformaldehyde was followed by 10 min in methanol at 4°C. Phospho-specific antibodies to p53 Ser-18P(Cell Signaling Technologies, Beverley, MA) were used with sections fixed with 80% methanol for 10 min at -20 CE, followed by 3 minute fixation with 2% paraformaldehyde and quenching with 0.1 M glycine in phosphate-buffered saline. After fixation, non-specific sites were blocked before addition of primary antibodies were incubated with sections for 1 h at room temperature (p53 Ser-18P) or overnight at 4C^B (LAP, TGF∃1, Smad 2/3) in a humidified chamber. Sections were washed in PBS containing 0.1% BSA, before incubating in secondary antibody conjugated to Alexa Fluor^{TM} 488 (Molecular Probes, Eugene, OR) for 1 h in a dark humidified chamber, washed and counterstained with DAPI (4=,6-Diamidino-2-Phenylindole, Sigma Pharmaceuticals, St. Louis, MO))e mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Apoptotic index: ApopTag (Intergen, Purchase, NY) was used for TUNEL staining and the supplied protocol followed with modifications. Briefly, fresh frozen sections were fixed in 1% paraformaldehyde, then in a precooled 2:1 ethanol:acetic acid mixture. Sections were blocked with the supernatant of 0.5% casein in PBS. The TdT stock solution was used at a working strength of 30% for 1 hour at 37 E C. The stop reaction and FITC anti_digoxygenin antibody steps were followed as written. Sections were counterstained with DAPI and mounted with Vectashield.

Image acquisition and analysis: Images were obtained using a 40x, 0.75 numerical aperture Zeiss Neofluar objective on a Zeiss Axiovert equipped with epifluorescence. A multiband pass dichroic mirror, barrier filter and differential wavelength filter wheel combination was used to selectively excite fluorochromes in sequence. Images were captured using a scientific-grade 12-bit charged coupled device (KAF-1400, 1317 x 1035, 6.8 μm square pixels) digital camera (Xillix, Vancouver, Canada). Images obtained from sections stained in parallel were captured with identical parameters and scaled using Scilimage (TNO Institute of Applied Physics, Delft, The Netherlands). False color images were compiled from gray-scale images of each fluorochrome.

TUNEL or PCNA positive cells were counted from at least four fields in each duplicate section from three mice or embryos. The frequency of apoptosis or proliferation was determined by counting the number of epithelial nuclei in each image. Nuclear counts in liver embryonic tissues were based on the total area of DAPI-stained nuclei divided by the mean area of 10 individually segmented nuclei. Statistical significance of differences

between genotypes was determined using the unpaired Student's t-test (GraphPad PRISM™).

Protein extraction and immunoblotting: Tissue extracts were prepared as previously described [Shyamala, 1990 #2273]. Equal amounts of protein lysates were run on reducing SDS-PAGE, immunoblotted and detected using a Pierce SuperSignal system (Pierce, Rockford, IL). Blots were also stained for total protein and probed for ∃-actin to assess equal loading. Exposed films or protein stained blots were scanned and subjected to densitometric analysis.

Results

TGF∃1 is reduced in mammary glands of irradiated *Tgf∃1* +/- mice.

In studies of mammary development, we determined that TGF∃1 protein levels are reduced by 90% in *Tgf∃1* +/- adult mammary gland [Ewan, 2002 #3421]. To confirm that radiation-induced TGF∃1 activation was also compromised, we localized active TGF∃1 by immunostaining. Active TGF∃1 (indicated by red/orange fluorescence) was greatly reduced in irradiated *Tgf∃1* +/- mammary gland compared to wildtype tissue (Figure 1A). To confirm that depletion of TGF∃1 resulted in decreased TGF∃1 signaling we then examined the induction of Smad 2/3 nuclear translocation in irradiated tissue. A marked induction of Smad 2/3 nuclear immunostaining was observed 1 h following radiation exposure in wildtype mice (Figure 1B). The frequency of positively stained cells and the intensity of staining was reduced in irradiated *Tgf∃1* +/- mammary epithelium. Thus, *Tgf∃1* +/- mice are an appropriate model to study whether TGF∃1 depletion affects cell fate decisions.

Radiation-induced apoptosis is absent in *Tgf∃1* +/- mammary epithelium.

Mammary gland is a quiescent tissue unless stimulated to proliferate and differentiate by ovarian hormones during puberty or pregnancy. As in other quiescent tissues like liver, the frequency of radiation-induced apoptosis is low compared to lymphatic tissues or actively proliferating epithelium, however the magnitude of the response is similar. Previous studies demonstrated that radiation induces a 2-3 fold increase in apoptosis that peaks at 6 h in mammary gland of nulliparous animals [Meyn, 1996 #3413; Kuperwasser, 2000 #2868]. During studies of mammary development, we observed that the background frequency of apoptosis is related to the stage of estrus cycle and that both proliferation and apoptosis peaks at estrus [Ewan, 2002 #3421]. Thus for radiation studies, the animals were irradiated while in estrus.

The apoptotic index increased 3-fold in mammary glands of C57BL/6/129Sv *Tgf∃1* +/+ mice 6 h following whole body exposure to a dose of 5 Gy (-radiation (Figure 2A). In contrast, mammary epithelial apoptosis was not significantly increased following irradiation of *Tgf∃1*+/- mice and was in fact 1/8th the level of irradiated wildtype mice. At estrus, physiological apoptosis in *Tgf∃1* +/- mammary epithelium is half that of wildtype mice [Ewan, 2002]. However, apoptosis is not generally depressed in *Tgf∃1* +/- mammary epithelium since levels are similar to wildtype at puberty and even increased relative to wildtype during pregnancy. In addition, radiation-induced apoptosis in lymph node and spleen was similar in *Tgf∃1* +/- mice and wildtype mice (not shown). These data suggest

that TGF∃1 affects cell fate decisions in response to DNA damage in a cell type-dependent manner.

Absence of TGF∃1 in embryonic tissues results in abrogation of apoptotic and cell cycle inhibition responses to IR.

It is not feasible to test the effect of TGF∃1 gene dosage following IR in adult mice since *Tgf∃1* -/- genotype mice commonly die *in utero* from defective placental vascular development [Dickson, 1995 #2246] or soon after birth from rampant inflammation [Letterio, 1994 #1867]. However, several embryonic tissues undergo either a robust apoptotic response or cell cycle inhibition shortly after radiation [Komarov, 1999 #2662]. Therefore 12.5 d pregnant *Tgf∃1* +/- dams were irradiated whole body with a dose of 5 Gy and the embryos collected 6 h later. TUNEL positive cells were counted in epidermis and liver (Figure 2 B,C). Apoptosis increased 2-3 fold in epidermis and liver in wildtype embryos following irradiation. Radiation-induced apoptosis was significantly decreased in *Tgf∃1* heterozygote embryos, and was absent in the embryos with a *Tgf∃1* null genotype.

In rapidly proliferating tissues, radiation can also induce a transient cell cycle block. Antibodies to PCNA were used to define the frequency of cells in cycle in embryonic tissues following IR (Figure 2 D,E). Proliferation was reduced 2-3 fold following irradiation in liver and epidermis of both wildtype and heterozygote embryos. However cell cycle blockade was absent in irradiated null genotype embryos. Togther, these data demonstrate that TGF∃1 abundance influences the cell fate decision of irradiated cells in both adult and embryonic epithelial tissues.

p53 stress response is activated in irradiated mammary gland.

Apoptosis in the irradiated mammary gland is p53 dependent [Meyn, 1996 #3413; Kuperwasser, 2000 #2868]. However, a recent report suggested that mammary gland lacks a classic p53 response as measured by total nuclear immunoreactivity using the CM5 antibody [Kuperwasser, 2000 #2868]. Since this antibody many be insensitive to activation status, in the current study we used a phosphorylation state-specific antibody. Phosphorylation of Ser-18 (Ser-15 in human) is strongly associated with the cellular response to radiation damage (e.g. apoptosis, cell cycle block) and contributes to p53 protein stability [Chao, 2000 #3386]. The phosphorylation of Ser-18 promotes dissociation of p53 from the MDM2 protein [Shieh, 1997; Siliciano, 1997], which otherwise directs p53 proteolysis [Haupt, 1997; Kubbutat, 1997]. Immunoblotting of total mammary gland protein extracts showed that Ser-18P was undetectable in extracts from unirradiated tissue but was induced within 1 h of radiation exposure (Figure 3A). p53 Ser-18P was evident to 24 h following radiation exposure. Total p53 levels, detected using antibodies PAb122 or CM1, which are insensitive to phosphorylation status, were increased at 24 h post-IR, but unchanged during the period from 1-15 h (data not shown).

Since the mammary gland is comprised of many cell types, we used immunofluorescence to determine the cellular localization of p53 bearing Ser-18P (Figure 3B). Mammary epithelium from sham-irradiated mice showed minimal nuclear signal. The immunoreactivity of phospho-specific p53 Ser-18P antibodies was restricted to the nucleus and was punctate in irradiated mammary epithelial nuclei. Epithelial nuclear p53 Ser-18P immunostaining was significantly increased within an hour of radiation exposure

and remained prominent up to 24 h after irradiation, which suggests that the signal for phosphorylation persists in irradiated tissue .

Induction of p53 Ser-18P is decreased in irradiated *Tgf∃1* +/- mammary gland.

Immunoblots of p53 Ser-18P using total protein extracts from wildtype mice showed a massive induction of p53 phosphorylation 1 h post-IR (Figure 4A). The level then decreased more than 10-fold between 1 and 6 h, but was still elevated compared to sham. In comparison, the p53 Ser-18p levels in irradiated *Tgf∃1* +/- mice compared to wildtypes were reduced by at least 4-fold at both 1 h and 6 h, suggesting that phosphorylation was persistently compromised. The immunoreactivity of p53 Ser-18P in irradiated *Tgf∃1* +/- mice was compared to that of wildtype mice (Figure 4B). There was a significant reduction of nuclear p53 Ser-18P immunofluorescence at 1 h post-irradiation in the *Tgf∃1* heterozygote mammary epithelium compared to wild type mammary gland. The difference between irradiated wildtype and *Tgf∃1* +/- mice was less pronounced at 6 h post-irradiation because p53 Ser-18P immunoreactivity decreased from 1 h to 6 h in wildtype mice, mirroring the response observed with immunobloting, but was still attenuated in the *Tgf∃1* +/- mice.

Transient TGF∃1 depletion inhibits p53 Ser-18 phosphorylation.

Chronic depletion of TGF∃1 in the null heterozygote mice could perturb aspects of cell physiology that modify the p53 radiation response. To test whether TGF∃1 directly affected the radiation response, pan-specific TGF∃1 neutralizing antibodies were administered i.p. 3 h before irradiation. Our previous studies had demonstrated the efficacy of this timing, route and antibody dose in blocking TGF∃1 mediated extracellular matrix remodeling [Ehrhart, 1997 #1879]. Similar to the results seen in the *Tgf∃1* +/- mice, nuclear localization of p53 Ser-18P determined by immunofluorescence staining was significantly reduced when TGF∃1 was transiently depleted prior to irradiation (Figure 5A).

Immunoblots of total mammary gland protein extracts from neutralizing antibody treated mice indicate reduction of p53 Ser-18P after radiation exposure (Figure 5B). p53 Ser-18P was reduced 5-fold in extracts from animals receiving TGF∃ neutralizing antibody at 1 h post-IR compared to those receiving control antibody. At 4 h post-IR, the p53 Ser-18P level was reduced compared to 1 h post-IR and were similar in animals treated with either TGF∃ or non-specific antibodies. Both sham and irradiated mice, regardless of TGF∃1 neutralizing antibody treatment exhibited similar levels of total p53, as detected using either CM1 or CM5 antibody (data not shown), indicating that TGF∃ affected p53 post-translational modification rather than absolute levels.

Discussion

The decision of a cell to undergo apoptosis in response to radiation is commonly attributed to the level of DNA damage and certain cellular competencies such as levels of pro- and anti-apoptotic proteins [Evans, 1993 #877]. The contribution of outside-in signaling from extracellular factors is poorly understood. The data reported here reveal a surprising dependence of intracellular cellular response to DNA damage on signaling from TGF∃1 originating extracellularly. We found that classic cellular responses to ionizing radiation, i.e. apoptosis and cell cycle block, are significantly reduced as a result of TGF∃1

depletion. The frequency of apoptosis in adult mammary epithelium and embryonic tissues was a function of gene dosage, while radiation-induced DNA synthesis block was absent only in *Tgfβ1* null embryo tissues. Thus two classic cellular responses to radiation depend on the level of TGFβ1. Our studies suggest that one mechanism by which TGFβ1 affects the cell fate decision in response to damage is through modification of the p53 response. Using Ser-18P as a marker of p53 activation, we determined that either chronic TGFβ1 depletion using the knockout model or transient depletion using TGFβ1 neutralizing antibodies resulted in reduction of IR-induced p53 phosphorylation in the mammary epithelium.

p53 responses are an important mechanism of tumor suppression that is underscored by the high frequency of cancer in Li-Fraumini syndrome, in which p53 malfunctions, and by studies in p53 knockout mice [Donehower, 1995 #2726; Jerry, 2000 #2866]. Further, mutant p53 is commonly found in human tumors and cancer cells [Runnenbaum, 1991 #994; Gerwin, 1992 #2118; Thompson, 1992 #1562]. The p53 stress response pathway is integral to tumor suppression via its action as a primary mediator of growth arrest and apoptotic responses to DNA damage. Activation of p53 in damaged cells may induce cell cycle progression delays expressed through either the production of G1/S or G2/M phase transition blocks that provide time for DNA repair [Kastan, 1991 #2589; Agarwal, 1995]. Alternatively, certain cells undergo p53-mediated apoptosis [Fisher, 1994 #2608]. The factors that influence which response occurs include the type of cell, the level of damage, and cell cycle status [MacCallum, 1996 #2665; Hendry, 1997 #2649].

Our hypothesis that TGFβ1 modulates the type and degree of cellular damage responses *in situ* suggests a complex interaction between cellular and extracellular sensors of radiation damage. Similarities between p53 and TGFβ1 regulation indicate that they are both equipped to participate in damage control. Both are abundant in latent forms that restrain activity. Rapid activation of the p53 stress response is predominantly post_translational. Covalent p53 protein modifications affect p53 stability and activity, which include phosphorylation, dephosphorylation, acetylation and deacetylation [Appella, 2001 #3463]. These modifications can, in turn, affect p53's binding partners, localization, activity and degradation [Momand, 2000 #3408]. Since the latent complex is abundant in bound and circulating forms, and all cells have TGFβ receptors, biological activity is controlled by extracellular processing that releases TGFβ from LAP. This activity is further modulated by binding to extracellular proteins such as thrombospondin [Murphy-Ullrich, 2000 #3503]. Both p53 and TGFβ1 exhibit redox sensitivity that endows them with the capability of being rapidly activated [Hainaut, 1993 #854; Barcellos-Hoff, 1996 #1871]. Both regulate complex cellular decisions regarding fate in response to insult, both are induced by a variety of damage and specifically ionizing radiation, and both undergo auto-regulatory translational and transcriptional control that moderate later events. These common properties enable both p53 and TGFβ to perform rapidly in response to significant DNA damage. However, intracellular p53 dictates individual cell fate, while extracellular TGFβ1 orchestrates diverse multicellular fates.

p53 status can affect responses to TGFβ1 and vice versa [Teramoto, 1998 #2625; Raynal, 1994 #2871; Wyllie, 1991 #2636]. The rapid induction of Smad 2/3 immunoreactivity that we observed in irradiated mammary tissue, and the observation that TGFβ1 enhances the stress response following ultraviolet irradiation [Merryman, 1998

#2630], suggests that there may be an interaction between the TGF∃ signaling and damage response pathways. It will be informative to determine whether there is a direct or indirect interaction between components the TGF∃1 signaling pathway (i.e. SMADs) and p53 that drives phosphorylation and thus stabilization of the p53 following IR.

Like IR [Barcellos-Hoff, 1994 #577], other DNA damaging agents induce TGF∃1 activation, including PALA [Glick, 1996 #2117], cisplatin [Ohmori, 1998 #2674] and alkylating agents [Yamada, 2001 #3441]. Studies using keratinocytes from *Tgf∃1* knockout mice also support a functional, rather than accessory, role for TGF∃ in damage response. PALA induced gene amplification was elevated more than 100-times in *Tgf∃1* null keratinocytes compared to wildtype cells, while exogenous TGF∃1 to knockout cells reversed instability [Glick, 1996 #2117]. Similar to our observations in irradiated *Tgf∃1* -/- embryos, *Tgf∃1* null keratinocytes lack the typical PALA-induced, p53 dependent G1 arrest.

Altered responsiveness to TGF∃1 has been broadly implicated in breast cancer [Wakefield, 2000 #2718; Massague, 2000 #2847]. We, and others, have argued that conversion to TGF∃1 growth resistance during breast cancer progression is a critical juncture in the evolution of malignant behavior [Reiss, 1997 #2121; Stampfer, 1997 #2266; Chen, 2001 #3391; Xie, 2002 #3501]. Indeed, at later stages of carcinogenesis TGF∃ can stimulate tumor progression [Derynck, 2001]. The correlation we have observed between decreased TGF∃1 expression and reduced p53 response in irradiated tissues suggests that TGF ∃1 should be considered as a key regulator of homeostasis following IR. Its early loss by whatever means could contribute to genome instability through reduced action of p53.

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Figure Legends

Figure 1: Irradiated *Tgf∃1* +/- mammary gland shows reduced levels of active TGF∃1 and Smad 2/3. *Tgf∃1* +/- and +/+ mice were irradiated whole body to a dose of 5 Gy and killed 1 h later. **(A)** False color digital micrographs of dual immunofluorescence of antigen-purified TGFβ1 antibody (red) and LAP antibody (green) visualized simultaneously with DAPI stained nuclei (blue). Comparison of mammary gland tissue from irradiated *Tgf∃1* +/+ and +/- mice indicates that TGF∃1 immunoreactivity (yellow-orange) is greater in *Tgf∃1* +/+ mice, while *Tgf∃1*+/- mice show predominant LAP immunoreactivity (green). The prominent localization of TGFβ1 in the irradiated wildtype mice reflects radiation-induced activation [Ehrhart, 1997 #1879]. Note that all cells stain with LAP. **(B)** False color digital micrographs of Smad 2/3 antibody (green) localized simultaneously with DAPI stained nuclei (blue). Comparison of mammary gland cryosections from sham (a, c) or irradiated (b, d) *Tgf∃1*+/+ (a, b) and +/- (c, d) mice indicates that radiation induced Smad2/3 immunoreactivity. The frequency of positive cells and intensity was reduced in irradiated *Tgf∃1* +/- mice.

Figure 2: *Tgf∃1* gene dosage correlates with reduced apoptosis and cell cycle block in response to radiation. (A) The frequency of apoptotic nuclei detected using TUNEL reaction was determined in the mammary epithelium of *Tgf∃1* +/- and +/+ mice (mean ± SEM; n= 3 animals). Sham-irradiated (black) and whole-body irradiated (gray) wildtype were significantly different (t-test; P=0.02). The irradiated *Tgf∃1* heterozygote mice were not significantly different from sham-irradiated heterozygote mice, but were significantly different from irradiated wild type (t-test; P=0.006). Pregnant NIH/OlaHsd *Tgf∃1* +/- dams were irradiated whole body (5 Gy) on day 12.5 of gestation. Embryos irradiated in utero were collected 6 h after irradiation. Apoptotic nuclei were detected using the TUNEL reaction in liver **(B)** and epidermis **(C)** from *Tgf∃1* +/+, +/- and -/- embryos. Apoptosis was decreased in control *Tgf∃1* +/- and -/- embryo tissues. Significantly increased apoptosis was absent from both liver and epidermis of irradiated *Tgf∃1* +/- and -/- embryos. The frequency of cycling cells was detected using PCNA antibodies in sham-irradiated (black) and irradiated (gray) embryos. Radiation induced cell cycle block was evidenced by a 2-3 fold reduction of PCNA positive cells following irradiation in utero in the liver **(D)** and epidermis **(E)** from *Tgf∃* +/+ and +/- embryos. The frequency of PCNA positive cells was not significantly different between sham and IR embryos of -/- genotype, indicating abrogation of radiation-induced cell cycle block.

Figure 3: p53 Ser-18P is induced in irradiated mammary epithelial cells. (A) Antibodies to Ser-18P p53 were used in Western blotting of total tissue protein extracts of irradiated Balb/c mammary tissue. No signal was evident in sham-irradiated tissue. A single band was detected at 1 h and was present up to 24 h following radiation exposure by Western. **(B)** False color images of immunofluorescence localization of p53 Ser-18 phosphorylation detected using secondary antibodies labeled with Alexa 488 (appears green/turquoise). Nuclei were counterstained with DAPI (blue). Immunofluorescence was absent from controls in which the primary antibody was deleted (a) and discernable in only

a few epithelial cells in sham irradiated tissue (b). Prominent nuclear immunoreactivity was evident throughout the epithelium from 1 h (c), 4 h (d), 15 h (e) and 24 h(g) after radiation exposure .

Figure 4: Radiation-induced p53 Ser-18 P is decreased in irradiated *Tgf∃1* +/- mammary epithelium. **(A)** Western blot of tissue extracts from wildtype or heterozygote mice sham and 1-6 h post-IR. p53 Ser-18 phosphorylation was significantly reduced in *Tgf∃1* +/- mice 1-6 h after IR. **(B)** p53 Ser-18P was localized as indicated in Figure 3 using cryosections of C57BL/6/129Sv *Tgf∃1* +/+ mice (a, b, c) or *Tgf∃1* +/- mice (d, e, f) subjected to sham exposure (a, d) or irradiated with 5 Gy, 1 h (b, e) or 6 h (c, f) before sacrifice. Nuclear localization of p53 Ser-18P 1 h post-IR was reduced in *Tgf∃1* +/- animals compared to wildtype animals. By 6 hr, p53 Ser-18P was decreased in both genotypes.

Figure 5: Radiation-induced p53 Ser-18P is decreased in mice treated with TGF∃1 neutralizing antibodies prior to radiation. **(A)** p53 Ser-18P was localized in tissue as indicated in Figure 3. Balb/c adult female mice were injected i.p. with an irrelevant IgG antibody as a control (a, b) or TGF∃ pan-isoform neutralizing monoclonal antibody (c, d) 3 h before sham exposure (a, c) or irradiation with 5 Gy (b, d). Nuclear localization of p53 Ser-18P was reduced in animals treated with TGF∃ neutralizing antibody. **(B)** Western blot of tissue extracts from animals that received control (C) or TGF∃1 neutralizing (N) antibody prior to irradiation. p53 Ser-18P was significantly reduced 1 h after IR when TGF∃1 neutralizing antibodies were administered before irradiation.